IN VITRO SCREEN FOR CYANIDE ANTIDOTES

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ABSTRACT

We previously reported that isolated rat pheochromocytoma (PC12) cells may be useful for in vitro evaluation of potential cyanide antidotes. The present study shows further results and in vivo validation of this in vitro approach. Ability to block a series of 6 biochemical markers of cyanide toxicity in PC12 cells (dopamine release, peroxide generation, cytosolic-free calcium) and inhibition of certain enzymes (catalase, superoxide dismutase and cytochrome oxidase) was evaluated for 39 different compounds from various pharmacological classes. Based on the composite scoring in all 6 assays, carbamazepine, mannitol, allopurinol and phenytoin were ranked as the most effective anticyanide compounds.

In an attempt to maximize in vitro protection, combinations of potential antidotes were used. However, combinations were less effective than antidotes used alone in in vitro assays. In some cases potential antidotes appear to interfere with each others actions in the in vitro screen.

Known cyanide antidotes (e.g., pyruvate, mercaptopyruvate, alpha-ketoglutarate, naloxone and flunarizine) obtained relatively high ranking in the PC12 cell screen. Furthermore a significant correlation was found between protective effects (based on LD_{50}s) of cyanide antidotes in mice and ranking in the in vitro screen. These results support the validity of the screen for cyanide antidotes using isolated PC12 cells. Several compounds have been identified which inhibit the biochemical actions of cyanide. These substances may be useful for enhancing effectiveness of the standard cyanide antidotes.

This work was supported by the U.S. Army Medical Research and Development Command under Contract DAMD17-89-9033.

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INTRODUCTION: The goal of the present work is to develop an in vitro screen for substances which block the biochemical actions of cyanide. Such a screen would require minimal use of animals for discovery of new anticyanide drugs. Substances effective in the screen may be useful in conjunction with standard cyanide antidotes (methemoglobin formers and sulfur donors) to enhance the treatment of cyanide intoxication.

Blockade of mitochondrial cytochrome oxidase with depletion of cellular energy stores has long been considered the primary biochemical effect of cyanide. However, recent reports showing an equivalent degree of cytochrome oxidase inhibition in brains and hearts of mice which died and those that survived doses of cyanide, cast doubt on this concept (1). Additionally, Yamamoto (2) showed that mice rendered unconscious by cyanide had no decrease in brain ATP whereas liver ATP decreased to 60% of control. Thus cytochrome oxidase inhibition may explain only part of the toxic syndrome caused by cyanide, and for this reason other biochemical actions of cyanide [elevated cytosolic calcium (3), peroxide generation (4) and inhibition of antioxidant defense enzymes (5)] were also used in the screen for antidotal activity.

METHODS: Rat pheochromocytoma (PC12) cells were obtained from American type culture collection (Rockville, MD) and grown (37°C, 5% CO_2 in humidified air) attached to plastic culture flasks in 85% RPMI 1640 medium (Gibco, Grand Island, NY) with 10% v/v heat-inactivated horse serum and 5% v/v fetal calf serum. At the time of the experiment, cells were detached using a stream of medium and suspended in Krebs-Ringer solution (KRB) consisting of (mM) NaCl, 124, Hepes-NaOH, 25; glucose, 6; $NaHCO_3$, 4; $MgSO_4$, 1.2; KH_2PO_4, 1.2 and $CaCl_2$, 1.0. Potassium cyanide solutions were prepared just prior to use. All potential antidotes were used in a concentration of 10 μM and were added to the samples 10 min before cyanide. The cyanide concentrations based on IC_{50} values were: 5 μM for cytochrome oxidase; 55 μM for catalase; 1 mM for SOD; 2 to 10 mM for cytosolic calcium. The following biochemical markers were measured: Cytochrome Oxidase Activity: Cytochrome oxidase activity, a primary target for cyanide, was determined spectrophotometrically by measuring the rate of oxidation of reduced cytochrome c at 25°C. Dopamine (DA) Release: Release of neurotransmitters by cyanide may be a critical aspect of the toxic syndrome and may explain many of the symptoms manifested after cyanide exposure. It was important therefore to test the effects of potential antidotes on cyanide-induced DA release from PC12 cells. DA released into the incubation buffer (KRB solution) of PC12 cells during 30 min incubation was estimated by HPLC (6). Free Intracellular Calcium: Prolonged elevation of free cytosolic calcium leads to cell damage through activation of proteases (7) as well as by other mechanisms. Inhibition of the increase in cell calcium may provide protection against cyanide-induced cell damage. Calcium-sensitive fluorescent dyes (quin-2AM or fura-2 AM) were used to measure cyanide-induced increases in cytosolic calcium. Catalase: Hydrogen peroxide is damaging to living systems, frequently because it gives rise to OH^0 radicals which can produce alterations in DNA, lipids and proteins in cells. It is therefore important that cells limit H_2O_2 accumulation. Catalase mediates the conversion of H_2O_2 to H_2O and O_2. The enzyme is distributed throughout the body and may play an important role in

limiting oxidative damage after cyanide exposure. The enzyme was measured according to the method of Abei (8). Superoxide Dismutase (SOD): CuZu superoxide dismutase, which is the cyanide sensitive form, is found in virtually all animal cells and serves to catalyse breakdown of superoxide (O_2^-) to H_2O_2 and O_2. This is an important reaction because it prevents interaction of O_2^- with iron in the cell and blocks formation of the highly reactive hydroxyl radical OH^o) (9). SOD activity in homogenates was estimated according to Oberly and Spitz (10). Peroxide Generation: Peroxides were assayed based on conversion of 2,7-dichlorofluorescein to the fluorescent form of 2,7 dichlorofluorescein by peroxides (8,9).

RESULTS AND DISCUSSION: The rationale behind the present work is that substances which block the biochemical effects of cyanide in isolated cells should protect against cyanide toxicity. Two findings support the validity of this approach. First, several known biochemical antidotes (e.g., naloxone, pyruvate, mercaptopyruvate and flunarizine) scored relatively high in the in vitro screen (11), and secondly, effectiveness in the screen is correlated with ability of selected compounds to prevent death due to cyanide in mice (Figure 1). Thus protection against cyanide in vivo was correlated with in vitro scores for chlorpromazine, naloxone, flunarizine and carbamazepine. Known cyanide antidotes which have mechanisms not operative in vitro (e.g., nitrate and thiosulfate) scored low in the screen as anticipated since the cells do not generate methemoglobin and have a low level of rhodanese for conversion of thiosulfate. Thus the screen appears to be capable of identifying biochemical cyanide antidotes, but would not be expected to detect sulfur donors or methemoglobin formers.

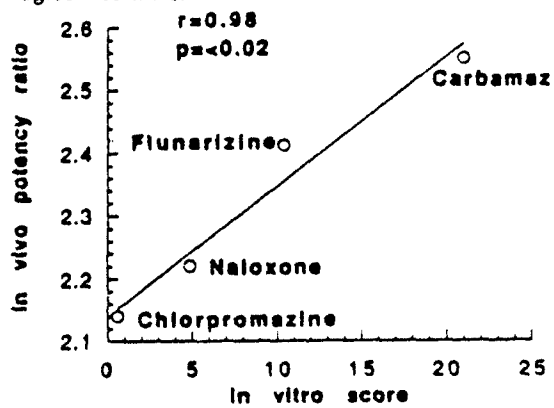

Figure 1. Correlation between in vitro and in vivo actions of cyanide antidotes. The potency ratio is the LD_{50} of cyanide divided into the LD_{50} of cyanide in mice pretreated with thiosulfate (1 g/kg ip, 15 min prior to KCN) and the antidote shown. Doses, routes and time intervals are as follows: chlorpromazine HCl 10 mg/kg sc, 30 min prior to cyanide; flunarizine 100 mg/kg ip, 1 hr before cyanide; naloxone HCl 20 mg/kg ip, 10 min prior to cyanide; carbamazepine 50 mg/kg ip, 1 hr before cyanide. The in vitro scores were determined in the PC12 cell system (11).

The anticonvulsants, phenytoin and carbamazepine were both effective in blocking cyanide-induced dopamine release. Of all the 39 compounds tested, only allopurinol, which was recently reported to have anticonvulsant effects itself (12), equaled the potency of the anticonvulsants. The main anticonvulsant action of phenytoin and carbamazepine is thought to be blockade of sodium channels to inhibit high frequency discharges around epileptic foci (13). Minimal disruption of normal neuronal traffic is produced by these drugs. These results suggest that cyanide may enhance sodium influx through channels responsible for high frequency discharges. In support, epileptiform discharges have been observed in guinea pig hippocampal slices after cyanide exposure in vitro (14).

Eleven additional substances beyond those given in a previous report (15) have been tested in vitro. One, trifluoperazine, was unique in partially protecting superoxide dismutase against cyanide. It also prevented cyanide-induced dopamine release to achieve a total protective score of 6.9 calculated as previously described (11,15). Since certain anticonvulsants were previously found to be effective in vitro and in vivo against cyanide, other anticonvulsants were also tested. Petit mal drugs, trimethadione, phenacemide and ethosuximide were not active and primidone and acetazolamide which are "broad spectrum" anticonvulsants were intermediate in effectiveness in the screen. Of the remaining substances tested, lidocaine and glyceraldehyde were most effective. Lidocaine inhibited dopamine release, peroxide generation and the rise in cytosolic calcium to achieve a score of 6.7. Glyceraldehyde also inhibited dopamine release and peroxide generation and protected catalase to a small extent to give a score of 8.3. Lidoflazine, promethazine, uric acid and hydroxytoluene had scores of 4.5 or less. It appears that grand mal anticonvulsants, channel blockers and aldehydes provide some protection of PC12 cells against cyanide in vitro.

To maximize protection against cyanide in vitro, compounds which were effective when used alone were combined with one another and employed to block the actions of cyanide. Combinations were selected to give as much protection as possible in each of the assays, e.g., a good antioxidant might be combined with a good blocker of dopamine release. After completing the whole range of 6 biochemical assays in vitro using 18 different combinations it was evident that the ranking scores were lower for many combinations than for the antidotes used separately. For example, carbamazepine alone gave a ranking score of 21.0 but when combined with pyruvate (which had a ranking score of 7.3) the resulting score was only 7.2. Apparently antagonism occurs in vitro when certain antidotes are combined in an attempt to block cyanide's biochemical actions.

Cyanide intoxication appears to be a complex syndrome and many factors contribute to the total cellular insult. Biochemical changes other than those measured in the present study may also contribute to the cellular damage. Nevertheless, the ranking scores generated from the in vitro data provide an empirical reflection of usefulness in vivo. It reasonable to suggest that substances which prevent cyanide-induced biochemical changes may be clinically effective as cyanide antidotes.

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